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Marking Otoliths by Immersion of Marine Fish Larvae in Tetracycline

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Abstract

Otoliths of the larvae of spot *Leiostomus xanthurus* and pinfish *Lagodon rhomboides* were marked with a band that fluoresced in ultraviolet (UV) light after live larvae had been immersed for 1–2 hours in tris-buffered, 100–500-mg/liter solutions of tetracycline in a 1% NaCl solution. Dimethyl sulfoxide did not significantly increase tetracycline uptake. After otoliths were marked, their radii increased approximately 18% in 8 days for spot and 15% for pinfish, but distinct daily growth increments could not be detected even though diel periodicities in feeding and photoperiod were provided in the laboratory.

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A common method of ageing larval fish is to count growth increments, or daily rings, on otoliths (Brothers et al. 1976; Rojas de Mendiola 1981; Warlen 1982). Validation of the rings as daily growth increments usually involves comparison of the actual age of larvae reared from eggs with the number of increments (Tanaka et al. 1981). When one works with larvae of unknown age, such as those collected in the field, validation of the ageing method is more difficult. A chemical time check, or mark, placed on the otolith can provide a reference point for subsequent timed growth (Campana and Neilson 1982).

Tetracycline is an in vivo bone-tissue marker and is detectable in undecalcified sections of bone by fluorescence microscopy (Milch et al. 1957). Calcium-containing tissues of fishes (vertebrae, scales, spines, otoliths) have been marked by injection of the fish with tetracycline (Jensen and Cumming 1967; Wild and Foreman 1980; Campana and Neilson 1982; Campana 1983) and by addition of this chemical to food (Choate 1964; Weber and Ridgway 1967). Choate (1964) was unable to mark hard parts of young brook trout *Salvelinus fontinalis* by immersion in tetracycline, but Campana and Neilson (1982) suggest that otoliths of starry flounder *Platichthys stellatus* can be marked by immersion in a tetracycline–saline solution. In this note, I describe a technique for mass-marking larval marine fishes by immersion in a tetracycline bath.

Methods

In a preliminary experiment, I marked otoliths of larval marine fish with a 75% propylene glycol solution containing 25 mg oxytetracycline per ml of solution. A glass needle connected to a micromanipulator was used to inject anesthetized larvae, 80–100 mg wet weight, with 0.1 µl of the solution. However, the procedure was slow and only about 10 larvae could be injected per hour.

For the experiment described in this note larval spots *Leiostomus xanthurus*, 13–16 mm SL (standard length), and pinfish *Lagodon rhomboides*, 12–15 mm SL, were caught in February near Beaufort, North Carolina, at a water temperature of 11 C and a salinity of 26‰. The flowing seawater in which larvae were held was raised in temperature to 20 C over 3 days and thereafter held at a constant 20 C. Ambient salinity ranged from 26 to 30‰ before and after tetracycline treatments. Groups of 15 larvae of each species were held in cylindrical 18 × 18 cm flow-through cages made of 2-mm-square monofilament nylon screen. Cages for all tetracycline treatments, including controls, were placed into a single water table containing flowing seawater 12 cm deep. Larvae were fed live *Artemia salina* nauplii and dry trout food once daily. Photoperiod was set on a 12-hour-light: 12-hour-dark cycle. Larvae were held 8 days following exposure to tetracycline.

For tetracycline treatment, all larvae in a cage were removed and placed in a 400-ml bath of 1% NaCl in aerated distilled water containing either 100, 250, or 500 mg/liter oxytetracycline hydrochloride. Sodium chloride was used instead of seawater because tetracycline chelates with calcium and magnesium, and then will not bind with calcium in bone-forming tissue. The 1% salt concentration is approximately isosmotic with marine fish tissues and less than one-third the osmotic pressure of seawater. Seawater-adapted juvenile pink salmon *Oncorhynchus gorbuscha* were marked best in freshwater tetracycline solutions (R. C. Johnsen, National Marine Fisheries Service, Rufus, Oregon, personal communication). However, I determined in preliminary tests that spot and pinfish larvae

TABLE 1.—*Mean percentages of spot and pinfish larvae with otoliths successfully marked by tetracycline. Each treatment had two replicates of five fish each. DMSO is dimethyl sulfoxide.*

Treatment		% marked			
		Spot		Pinfish	
Tetracycline (mg/liter)	Exposure (minutes)	With DMSO	Without DMSO	With DMSO	Without DMSO
100	30	0	0	0	0
	60	0	0	0	0
	120	60	0	0	0
250	30	0	0	0	0
	60	50	50	0	0
	120	100	100	0	0
500	30	70	0	0	0
	60	100	90	20	30
	120	100	100	100	100

could not tolerate fresh water, so 1% saline was used. Half of the treatments contained 2% dimethyl sulfoxide (DMSO), a solvent that may increase the uptake of tetracycline (R. C. Johnsen, personal communication). Each tetracycline bath, with and without DMSO, was adjusted to pH 6.0–6.2 with tris buffer. The pH of unbuffered tetracycline solutions varied with concentration from 2.5 to 3.5 and was lethal to the larvae. After exposures of 30, 60, or 120 minutes, the larvae were returned to their cages. Two replicates of each treatment were made, resulting in 36 treatments and four controls for each species. Controls were groups of fish exposed to 1% NaCl in aerated distilled water for 120 minutes and then returned to flowing seawater.

All larvae surviving 8 days after treatment were preserved in 95% ethyl alcohol. Sagittae were extracted from five specimens of each species from each treatment replicate and mounted, with the flattest surface down on glass microscope slides, with cyanoacrylate adhesive. Astericus and lapillus otoliths were not used. Sagittae were then ground flat to the margins on the convex side with 600-grit wet–dry silicon carbide paper and polished with 1-μm diamond polishing compound. For grinding, the abrasive paper was glued flat to a glass slide with contact cement and two 10-μm spacers of waterproof tape were attached parallel to the edges of the slide. The mounted otoliths, approximately 30–40 μm thick, were ground down to the spacers against the abrasive with water as a lubricant, resulting in 10-μm sections. During grinding, care was taken not to grind away the otolith edge. Sectioned otoliths were examined microscopically with incident UV (ultraviolet) light at 100× and 560× magnification for the presence of a fluorescent mark.

Results and Discussion

In the more concentrated solutions and at the longer exposure times, tetracycline was absorbed by larvae of both species and produced fluorescent bands on the otoliths (Table 1; Fig. 1). Spots deposited detectable amounts of tetracycline at lower exposure concentrations and times than did pinfish. Pinfish failed to exhibit fluorescence except at the highest concentrations. The mark fluoresced yellow in contrast to the green autofluorescence observed around the margins and contours on the unground surface of the otolith.

The overall survival rate of spot larvae exposed to tetracycline was 93.5%, 8 days after marking. Spot controls, held in cages without exposure to tetracycline baths, had a survival rate of 96.5%. No pinfish died during marking or rearing. The mean survival rate of spot larvae in immersion baths without DMSO was 95.7% compared to 91.3% in baths containing 2% DMSO. Dimethyl sulfoxide did not affect the survival rate (t-test on arc sine-transformed data; $P = 0.255$). The use of 2% DMSO was based on a preliminary survival test in which larvae were exposed to concentrations of 0–10% DMSO for 1 hour, following the personal communication of Johnsen, who marked juvenile salmon in concentrations of 0, 4, and 10% DMSO. If DMSO is used, the baths must be cooled before larvae are introduced, because the solvent has a high heat of solution.

Although tetracycline fluorescence is known

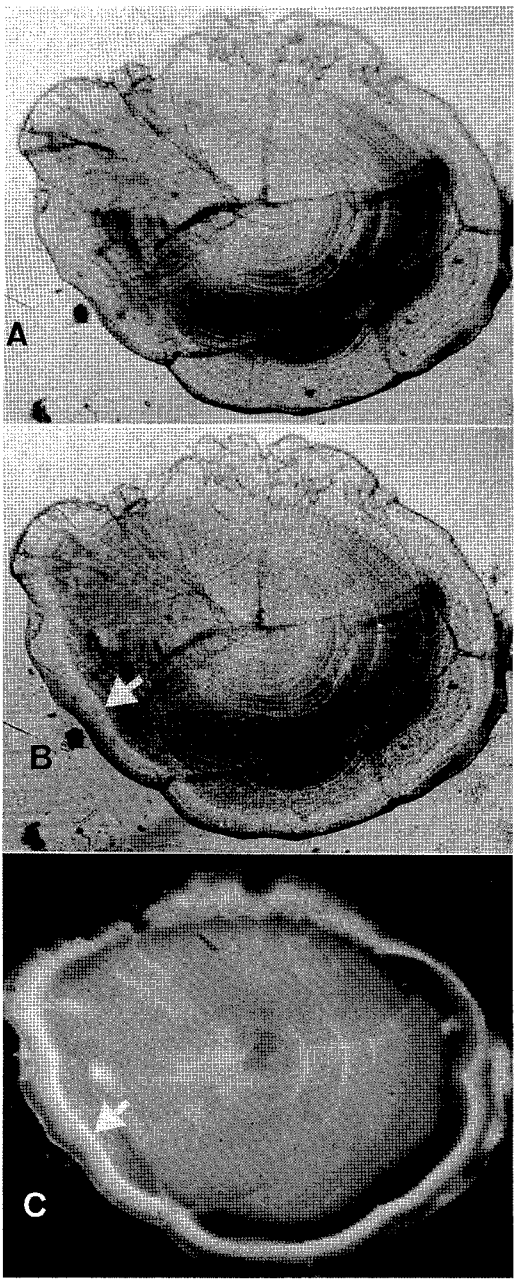

FIGURE 1.—*Section of 102-μm diameter sagitta from a 15-mm standard length spot larva that had been immersed 120 minutes in a saline solution of 500 mg/liter tetracycline without dimethyl sulfoxide. A—transmitted white light; B—combination of transmitted white light and incident ultraviolet (UV) light; C—incident UV light only. Arrow marks fluorescent band on B and C.*

to be photolytic in the presence of strong light, otoliths preserved in intact larvae for 6 months in 95% ethyl alcohol and held in the dark retained marks, as did sections of otoliths mounted on glass slides for the same interval.

In the 8 days after marking, eight daily rings should have been deposited on the otoliths between the mark and the margin, but the number deposited could not be accurately determined. Growth did occur, because otolith radii increased approximately 18% in spots and 15% in pinfish. Increments could be detected from the primordium out to about two-thirds of the otolith radius (Fig. 1), but increments beyond the fluorescent mark on most otoliths could not be clearly determined, perhaps due to experimental stress on the larvae. On a few otoliths, three or four irregularly spaced increments could be counted distal to the mark. Control fish, which were exposed only to saline solution and then held in seawater, also did not have detectable outer increments. Lack of distinct increment formation may have been due to laboratory conditions that did not create a strong diel pattern of growth. Geffen (1982) found that the mean ring deposition rates in larvae of turbot *Scophthalmus maximus* ranged from 0.07 to 1.0 per day, depending on feeding conditions and photoperiod, and that larger larvae had more rings than smaller larvae of the same age and from the same rearing condition. Campana (1983) found that increment deposition in tetracycline-marked starry flounder was variable, and ceased altogether in some fish. Tanaka et al. (1981) did find a daily rhythm of growth responding to laboratory photoperiod in *Tilapia nilotica* otoliths examined with a scanning electron microscope. The need for detection of a fluorescent marker precludes the use of scanning electron microscopy (SEM), although Campana (1983) recently described his procedure for comparing SEM photographs with paired UV–bright-field micrographs.

This study shows that a fluorescent marker can be placed easily on otoliths of marine fish larvae by immersion in tetracycline. For stenohaline marine larvae, the immersion baths may need to be a 3.5% NaCl saline solution to prevent death from osmotic stress. The applied mark may serve as a time mark for validation of otolith increment-deposition rates, if suitable

conditions for normal otolith formation are provided.

Another potentially useful benefit of immersion marking was observed when preserved specimens were placed under longwave UV light. Heads and fins, especially caudal fins, fluoresced yellow, making it possible to sort marked from unmarked larvae. Larvae marked with 100 mg/liter of tetracycline fluoresced much less noticeably, however, than larvae marked with 500 mg/liter. Unmarked larvae autofluoresced pale green over the entire body. Mass marking of marine fish larvae for mark–recapture studies seems useful, provided the longevity of the external fluorescence is known. Large populations could be marked without individual handling and there would be no visible marks to affect survival.

References

BROTHERS, E. B., C. P. MATHEWS, AND R. LASKER. 1976. Daily growth increments in otoliths from larval and adult fishes. United States National Marine Fisheries Service Fishery Bulletin 74: 1–8.

CAMPANA, S. E. 1983. Feeding periodicity and the production of daily growth increments in otoliths of steelhead trout (*Salmo gairdneri*) and starry flounder (*Platichthys stellatus*). Canadian Journal of Zoology 61:1591–1597.

CAMPANA, S. E., AND J. D. NEILSON. 1982. Daily growth increments in otoliths of starry flounder (*Platichthys stellatus*) and the influence of some environmental variables in their production. Canadian Journal of Fisheries and Aquatic Sciences 39:937–942.

CHOATE, J. 1964. Use of tetracycline drugs to mark advanced fry and fingerling brook trout (*Salvelinus fontinalis*). Transactions of the American Fisheries Society 93:309–311.

GEFFEN, A. J. 1982. Otolith ring deposition in relation to growth rate in herring (*Clupea harengus*) and turbot (*Scophthalmus maximus*) larvae. Marine Biology 71:317–326.

JENSEN, A. C., AND K. B. CUMMING. 1967. Use of lead compounds and tetracycline to mark scales and otoliths of marine fishes. Progressive Fish-Culturist 29:166–167.

MILCH, R. A., D. P. RALL, AND J. E. TOBIE. 1957. Bone localization of the tetracyclines. Journal of the National Cancer Institute 19:87–93.

ROJAS DE MENDIOLA, B. 1981. Daily rings in otoliths in larval anchovy (*Engraulis ringens*). Rapports et Procès-Verbaux des Réunions, Conseil International pour l'Exploration de la Mer 178:565–566.

TANAKA, R., Y. MUGIYA, AND J. YAMADA. 1981. Effects of photoperiod and feeding on daily growth patterns in otoliths of juvenile *Tilapia nilotica*. United States National Marine Fisheries Service Fishery Bulletin 79:459–466.

WARLEN, S. M. 1982. Age and growth of larvae and spawning times of Atlantic croaker in North Carolina. Proceedings of the Annual Conference Southeastern Association of Fish and Wildlife Agencies 34:204–214.

WEBER, D., AND G. J. RIDGWAY. 1967. Marking Pacific salmon with tetracycline antibiotics. Journal of the Fisheries Research Board of Canada 24:849–865.

WILD, A., AND T. J. FOREMAN. 1980. The relationship between otolith increments and time for yellowfin and skipjack tuna marked with tetracycline. Inter-American Tropical Tuna Commission Bulletin 17:509–560.